In vivo evaluation of corneal wound healing in superficial keratectomy with the tandem scanning confocal microscope

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Abstract

Aim: An *in vivo* study with a tandem scanning confocal microscope (TSM) on the process of keratocyte activation, proliferation, and stromal fibrosis after superficial keratectomy, and nylon suture penetration of rabbit cornea.

Materials and methods: A TSM which would provide improved resolution, magnifiction and contrast, as well as serial optical sectioning ability, was used for real-time examination of the rabbit cornea following superficial keratectomy. Additionally, the effects of nylon suture penetration and stromal revascularization were studied.

Results: Despite an apparently stable slit lamp appearance, with minimal subeoithelial fibrosis, the TSM revealed an actively evolving process of collagen remodelling in the anterior stroma. A suture placed in the stroma was also observed to produce significant tractional forces and tissue displacement. Subsequent stromal fibroblast activation and fibrosis around the nylon was also observed.

Conclusion: Our results suggest that TSM is a useful and effective instrument in evaluating corneal stromal wound healing at the cellular level.

Introduction

In recent years, many new instruments have been developed to image the eye. Utilizing different physical and optical principles, ranging from ultrasound, doppler echography, to laser confocal optics, each is optimally suited to visualize selective parts of the globe. In the cornea, the specular microscope has found its place alongside the venerable slit lamp as a 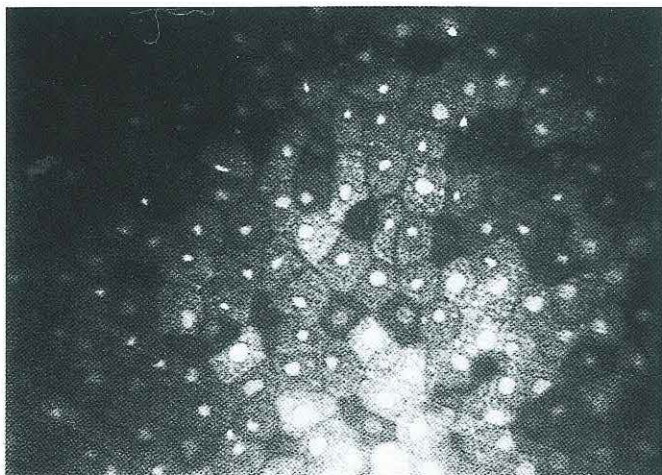modality to evaluate this transparent tissue. More recently, *in vivo* tandem scanning confocal microscopy (TSM) has been applied to the study of the anterior segment.[1-4] With its superior vertical and lateral resolution, contrast, and optical sectioning ability,[5-8] this instrument has been shown to provide the clinician with histologic views of otherwise clear tissues in real-time. We used the tandem scanning confocal microscope (TSM) to investigate the pathogenesis of various corneal conditions, including infectious keratitis,[9,10] chemical toxicity,[11] organic foreign body trauma,[12,13] and excimer laser photoablation.[14] In these studies, the TSM proved capable of resolving corneal stromal details at the cellular level, often despite the presence of opacities and edema.

Corneal wound healing is one of the main challenges facing the clinician. The management of wounds from various causes, including trauma, chemical burns, or iatrogenic injury, requires meticulous and frequent examination and careful documentation of the findings.[15] The evaluation of treatment efficacy with an ever increasing variety of drugs, and the early detection of the onset of complications such as infection and excessive inflammation also demand an objective method for examining and recording our observations. Superficial keratectomy is one such source of iatrogenic trauma, in which a successful surgical outcome is totally dependent on controlled wound healing. Used for the excision of pterygia, and as a component of other operations such as lamellar keratectomy, and refractive keratomilieusis, it requires careful post-operative monitoring to assess keratocyte activity, inflammation, and infection.

In this study, we used the TSM to examine the rabbit cornea *in vivo* during wound healing following superficial keratectomy. By sequential examination at high magnification, we were able to trace the evolution of keratocyte proliferation, stromal fibrosis, and collagen remodelling in this model of wound healing. Additionally, the effects of nylon suture penetration, and stromal neovascularization were studied.

Figure 1. *In vivo* confocal view of the surface epithelial cells of a normal rabbit cornea. The borders of individual epithelial cells were highly refractile as were their nuclei.
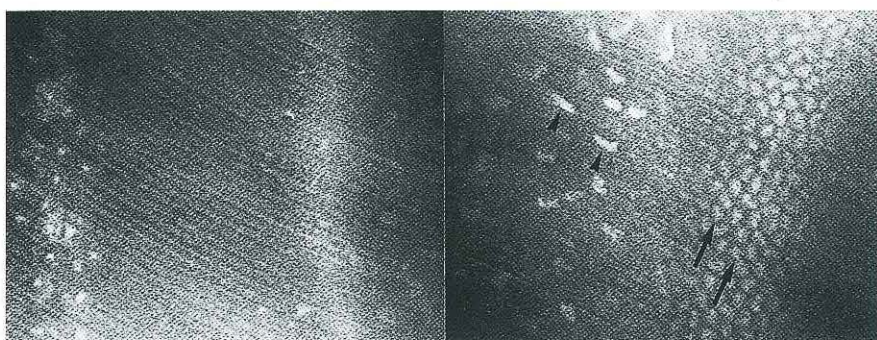

Figure 2. The three layers of the rabbit cornea as seen with the TSM. In the cross sectional view of the epithelium, only the superficial epithelium and the anterior stromal keratocytes were seen. The nuclei of stromal keratocytes appeared as elongated nuclei 5 μm in length (see arrow heads). Endothelium was visible in a broad field of 200 x 300 μm (see arrows).

Materials and methods

Corneal wounds in the rabbit

Ten New Zealand white rabbits were anesthetized with an intramuscular injection of keramine hydrochloride (40 mg/kg) and xylazine (5 mg/kg). Topical proparacaine was instilled in the conjunctival sac. A superficial keratectomy was performed with a 6 mm trephine advanced to a depth of 100-200 μm. The epithelium and anterior stromal lamellae were then removed as a single sheet using Colibri's forceps. In selected animals, 9/0 nylon sutures were placed in the mid-peripheral stroma to promote corneal neovascularization. The sutures were placed under the control of an operating microscope and assessed to penetrate at least 2/3 the stromal depth, without perforation into the anterior chamber. No topical antibiotics or steroids were administered postoperatively.

Slit lamp and confocal microscopic examination were performed immediately after the procedures, and at twice-weekly intervals until wound healing was judged to be complete and stable after 3-4 weeks.

Confocal microscopic examination

The animals were lightly anesthetized and corneal examination was performed with the aid of an eyelid speculum. An optically reconfigured TSM (Noran, Middlefield, WI) was used. The dual light path instrument has a ceramic Nipkow-type disc with 14,000 60-μm pinholes revolving at 1500 rpm. This permits 1% light transmittance. Illumination was supplied by a 200 Watt mercury lamp. The front

dichroic mirrors of the instrument were realigned for upright viewing, to duplicate the position used in slit lamp observation. The eyes were optically coupled to a X 25 water immersion objective lens (numerical aperture = 0.8, working distance = 1 mm) with methylcellulose. Images were then obtained with a video camera (Model CCD 200 E, Video Scope International, Washington DC) and digitized with an image analysis program (Optimas, Bioscan Inc, Edmonds, WA) using an IBM-PC compatible personal computer. An S-VHS video recorder was also used to record the examination. This combination of data and image storage permitted on-line image enhancement and off-line analysis. Hard copies of images were produced on a video printer.

Results and observations

Normal cornea

Figure 1 shows the *in vivo* confocal view of the surface epithelial cells of a normal rabbit cornea. The borders of individual superficial epithelial cells were highly refractile as were their nuclei. The three layers of the rabbit cornea as seen with the TSM are shown in Figure 2. In the cross sectional view of the epithelium, only the superficial epithelium and the anterior stromal keratocytes were seen. Basal and wing cells were not visible *in vivo*. The nuclei of stromal keratocytes appeared as elongated nuclei 5 μm in length. The cell borders were not apparent until the onset of cell activation. Endothelium was visible in a broad field of 200 x 300 μm.

Wound healing in superficial keratectomy

Immediately after wounding, the roughness of the underlying stroma was readily appreciated by TSM as collagen fibers in disarray to a depth of 100 μm. This combined epithelial and superficial stromal defect was accompanied by the degeneration of keratocytes in the subjacent stroma.[16] The disappearance of these cells was preceded, in the first 6 hours, by an increased density on TSM, followed by a rounding-up of the ovoid nuclei to a condensed 1-2 μm particle. By 12 hours, the anterior stroma was devoid of keratocytes. No changes in keratocyte morphology was found in the posterior stroma.

The stromal defect was also partially filled by thickened epithelium initially (Figure 3). In these areas, basal cells and apparent wing cells
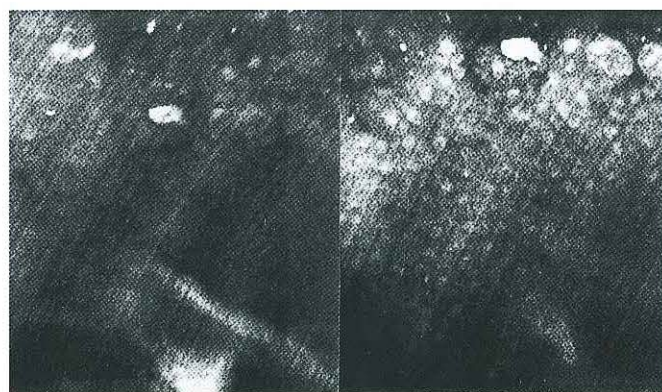

Figure 3. The stromal defect was partially filled by thickened epithelium initially. In these areas, basal cells and apparent wing cells were visible throughout the depth of the stratified epithelium. Isolated fibers of disordered collagen lamellae were interspersed within these regions.
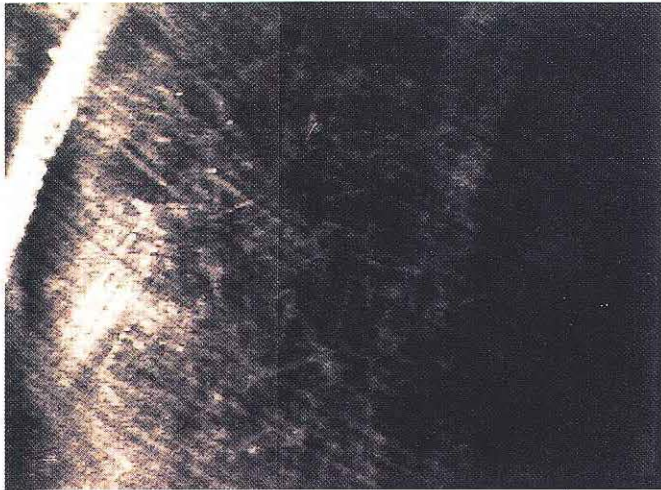
Figure 4. As epithelial wound closure progressed, the anterior stromal keratocytes increased in number, taking the appearance of elongated spindles 10-25 μm long.

Figure 6. A more homogeneous network of collagen lamellae was seen at four weeks after keratectomy.

Figure 5. The thin fibrils evolved into thick cords of up to 100 μm in diameter, performated by relatively clear interspaces at two weeks after keratectomy. The appearance of these regularly arranged fibrous bundles was coincident with the subepithelial haze seen on slit lamp examination.
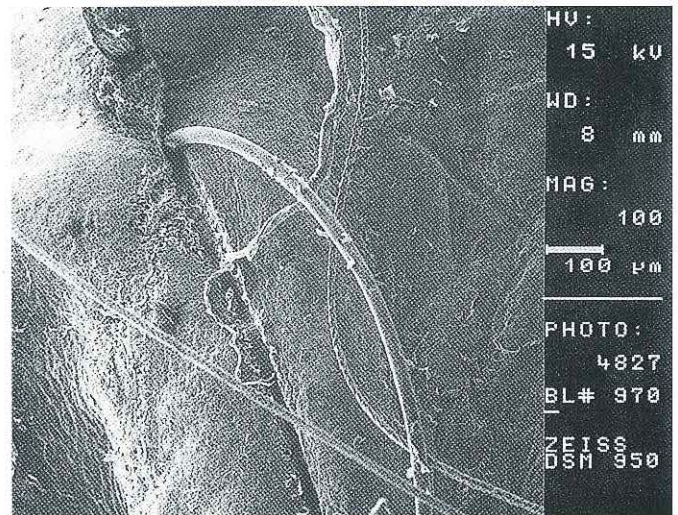
Figure 7. The scanning electron microscopic appearance of the suture following fixation.

were visible throughout the depth of the stratified epithelium. Isolated fibers of disordered collagen lamellae were interspersed within these regions. As epithelial wound closure progressed, the anterior stromal keratocytes increased in number, taking the appearance of elongated spindles 10-25 μm long. These were present in greatest numbers in the superficial 100-200 μm of the stroma (Figure 4). These activated keratocytes were later indistinguishable from the concurrently accumulating intercellular material which presented as elongated fibrils of 50 to 250 μm in length. Present from the second post-operative day, this finding may represent the nascent collagen fibers. As classical collagen fiber deposition is usually documented at a later stage, an alternative explanation of their nature may be the normally non-refractile mucopolysaccharides, keratin and chondroitin sulfate, secreted by the keratocytes. Histochemistry has not yet been performed to confirm the nature of these formations.

Fibroblast proliferation and collagen remodelling was reviewed regularly to monitor the wound organization. After 2 weeks, the thin fibrils
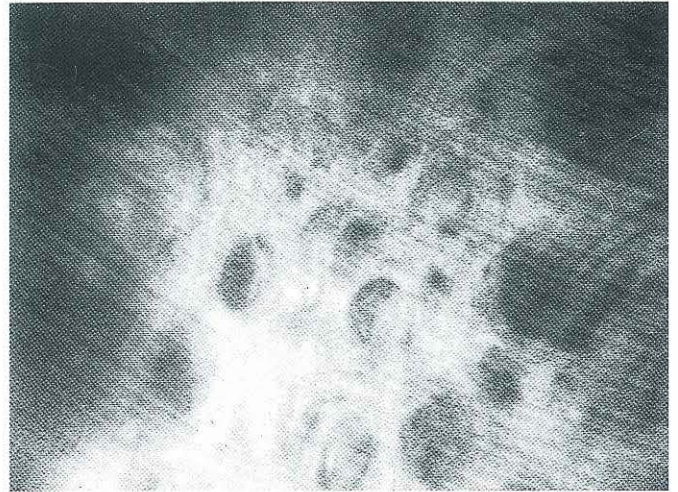
evolved into thick cords of up to 100 μm in diameter, performated by relatively clear interspaces (Figure 5). The appearance of these regularly arranged fibrous bundles was coincident with the subepithelial haze seen on slit lamp examination. No distinct cell nuclei were visible at this stage. This was gradually remodelled over the ensuing two weeks to a more homogeneous network of collagen lamellae, along which keratocyte nuclei were seen (Figure 6). Vestiges of this distinctive morphology of unorganized collagen persisted even at six weeks despite the apparent optical clarity of the cornea on slit lamp biomicroscopy. No changes in the endothelium were found throughout the observation period, nor was stromal inflammation apparent.

Nylon suture foreign body reaction

A separate experiment was performed to investigate the stromal changes following the placement of a 10/0 nylon suture in the mid-peripheral cornea. Figure 7 shows the scanning electron microscopic appearance of the suture following fixation. Preparative sectioning and shrinkage caused gross distortion artefacts which prevented any

Figure 8. Tractional effects (see arrows) of the nylon in the stroma *in vivo* with TSM.
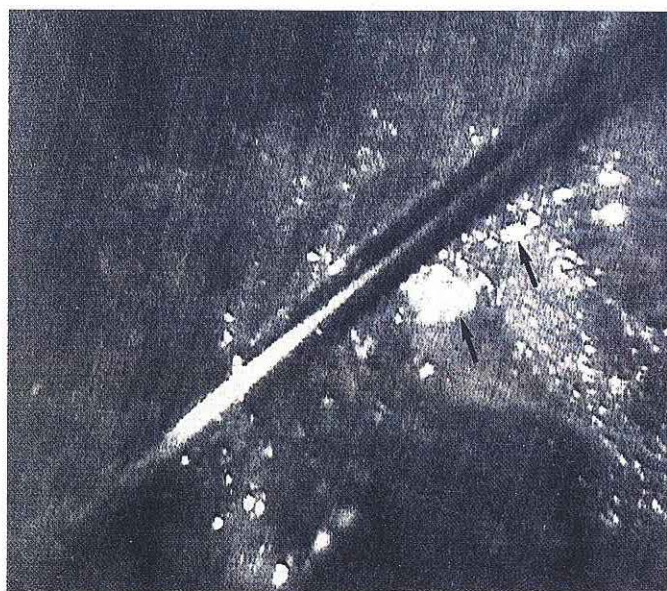

Figure 10. Long slender (1 μm) thread-like extensions (see arrows) from the keratocytes were present within two hours in contact with the suture.

Figure 9. The fibrin exudate aggregation (see arrows) following the introduction of the foreign body.

Figure 11. Cross-sectional perspective of the stromal neovascularization. Diapedesis of leukocytes which became adherent to the vessel walls could be demonstrated (arrow).

interpretation of the changes in the tissue as a result of suture placement. The suture was now separated from its groove in the endothelium, with consequent tissue damage. In contrast, we were able to demonstrate tractional effects of the nylon in the stroma *in vivo* with TSM (Figure 8). Even the gentle indentation of the cornea by the objective lens during the examination induced a substantial movement of the suture within its track. The fibrin exudate following the introduction of the foreign body could be appreciated (Figure 9). Long slender (1 μm) thread-like extensions from the keratocytes were present within two hours in contact with the suture. Over the ensuing 48 hours, a denser aggregate of fibrin and collagen fibrils was formed (Figure 10). No inflammatory cells were discernible. The slit lamp did not reveal any significant opacity around the suture.

Stromal neovascularization was observed in one of the corneas where inflammation developed in a suture whose knot was deliberately exposed (Figure 11). TSM provided a cross-sectional perspective of the vessels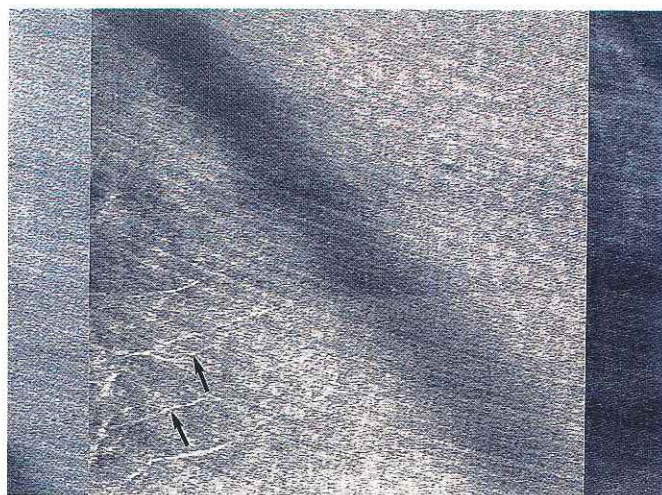. In adequately stabilized preparations, we monitored diapedesis of leukocytes which became adherent to the vessel walls. These changes were previously described by our laboratory in monitoring experimental corneal graft rejection *in vivo*.[17]

Discussion

Many authors have studied the natural history of corneal wound healing.[18] However, the traditional techniques of investigation, such as conventional light microscopy and electron microscopy, are limited to providing us with static images of the dynamic and rapidly evolving events in this tissue. Furthermore, fixation artefacts may blur the distinction between degeneration and regeneration. We found that the TSM offered a suitable modality for investigating the complex events of stromal healing sequentially *in vivo*, with a minimum requirement for experimental animals.

Healing was much slower centrally than peripherally, a characteristic attributable to the avascular nature of the cornea. Controversies exist

as to whether the cells are produced centrally by transformed dividing keratocytes, or whether they migrate in as mononuclear cells from the limbal tissue, or whether they appear from the aqueous in penetrating wounds. While it has been claimed that 75-80% are apparently transformed at the wound margin,[19,20] we found that substantial keratocyte proliferation occurred from the deep stroma. We were unable to demonstrate a gradient of increasing fibroblast density from the center of the wound to its edge, as predicted by the limbal migration model of keratocyte regeneration. It is our impression that the confidence of keratocyte quantification by TSM optical sectioning surpasses the accuracy achieved by the use of histologic sections.

While we are familiar with the slit lamp appearance of corneal wound healing, this study illustrates the new visage which TSM offers to complement our post-operative evaluations. With it, we achieved a higher resolution in the description of normal and aberrant migration and the proliferation of keratocytes. By defining the nature of stromal haze at the cellular level we are better able to detect the onset of inflammation and to discern the effects of growth modulating agents. ◉

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